Glycolipids and other lipid constituents of normal human liver

PETER O. KWITEROVICH, JR., HOWARD R. SLOAN, and DONALD S. FREDRICKSON

Molecular Disease Branch, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT An analysis of the lipids in normal human liver is presented which is particularly designed to assist in the classification and study of lipid-storage diseases. Special emphasis has been given to a determination of the quantity and composition of the neutral glycolipid classes and predominant ganglioside (G_{M3}). The neutral glycolipid content of 0.19 (SD ± 0.11) μmoles per g wet tissue, represented 0.4% of the total lipid in liver. Ceramide dihexoside was the most abundant neutral glycolipid. The mean contents of cholesterol, glycerides, and total phospholipids were 3.9, 19.5, and 25.1 mg/g wet weight, respectively. The relative amounts of seven different phospholipid classes were also determined; these included cardiolipin (diphosphatidylglycerol), which constituted 3.9% of the liver phospholipids.

SUPPLEMENTARY KEY WORDS cholesterol · triglyceride · phospholipid · neutral glycolipids · gangliosides · hematoside · cardiolipin · sphingomyelin

As THE NUMBER of recognized human lipid-storage diseases (lipidoses) increases, so does the need for more comprehensive analyses of tissue lipid content. Of at least 15 different phenotypes recognizable among these disorders (1), only those known collectively as Gaucher's disease can be diagnosed with reasonable accuracy without quantitative measurements of organ lipid content. The latter are still required even though enzymatic analyses are now becoming useful adjuncts to diagnosis. By helping to expose variants among disorders once considered homogeneous, enzyme measurements have actually increased the need for a more complete search for chemical heterogeneity in affected tissues.

The organ most commonly available for antemortem diagnosis through biopsy is the liver. This tissue has been used extensively to ascertain abnormal accumulation of cholesterol, triglycerides, sphingomyelin, and glucocerebrosides, but the potential for demonstrating hepatic storage of other glycolipids in the presence of a generalized defect in their metabolism has never been adequately exploited. One drawback to this has been a lack of adequate information concerning the normal content and composition of liver glycolipids; these have been the subject of only one previous and relatively brief report (2). We have adapted for diagnostic purposes a sequential analysis of the liver lipids which includes the glycolipids. The latter have been characterized, and their content has been measured in a series of normal livers. The results are described in this report. For purposes of reference and comparison with the glycolipids, the content of all other major lipid classes in these livers is also presented.

MATERIALS

Tissues

Samples of liver were obtained at autopsy from 12 different patients, 4–61 yr of age, who died from causes unrelated to liver or lipid-storage diseases. Sections of each liver were examined by light microscopy after routine histological preparation, and judged to be

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; Glc, glucose; Gal, galactose; Cer, ceramide; GalNAc, *N*-acetylgalactosamine; NANA, *N*-acetylneuraminic acid; NPL, nonpolar lipid; FFA, free fatty acid; GL-1, glucosyl ceramide; GL-2, galactosylglucosyl ceramide; GL-3, galactosylgalactosylglucosyl ceramide; GL-4, *N*-acetylgalactosaminylgalactosylgalactosylglucosyl ceramide; G_{M3}, *N*-acetylneuraminic acid galactosylglucosyl ceramide (hematoside); LysoPC, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; CL, cardiolipin.

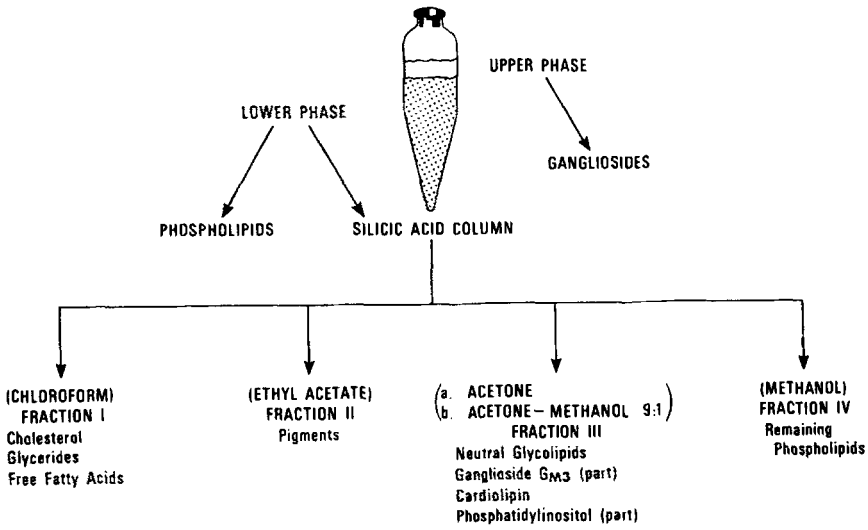

FIG. 1. The sequential system for liver lipid analysis employed. From 0.5 to 1.0 g of human liver were extracted and partitioned according to the method of Folch et al. (10). The lower phase was separated on a silicic acid column into the four fractions shown. The compositions of phospholipids and gangliosides were determined separately on a small aliquot of the lower or upper phases.

morphologically normal by a pathologist. Some specimens were extracted immediately; others were immediately stored in airtight containers at −70°C for 1–30 months before extraction.[1]

Other Materials

Unisil (silicic acid) 200–325 mesh, was obtained from Clarkson Chemical Co., Inc., Williamsport, Pa., and Amberlite anion exchange resin CG-4B was bought from Mallinckrodt Chemical Works, St. Louis, Mo. Diethylaminoethyl cellulose, microgranular DE 52, was purchased from H. Reeve Angel, Inc., Clifton, N. J. Analytical grade cation exchange resin AG50 W-X8, was obtained from Bio-Rad Labs, Richmond, Calif. Uniplates (Silica Gel G-R2A)-250 μ were purchased from Analtech, Inc., Wilmington, Del. TLC plates of Silica Gel F-254 were obtained from Brinkmann Instruments Inc., Westbury, N. Y. All solvents were of the highest purity commercially available and were not redistilled. All gases for GLC were high purity grade and were obtained from Air Products and Chemicals, Inc., Washington, D.C. SE-30 3% on Gas-Chrom Q, 80–100 mesh (lot No. SP-546) was purchased from Applied Science Laboratories Inc., State College, Pa. EGS 15% on Chromosorb W HI-EFF-2BP, 80–100 mesh (lot No. 7132) was secured from Applied Science Laboratories, Inc. Certified silver carbonate was obtained from Fisher Scientific Company, Pittsburgh, Pa. Synthetic *N*-lignoceryl DL-sphingosine β-D-lactoside (cytolipin H), synthetic *N*-lignoceryl DL-sphingosine β-D-galactoside (kerasin), and synthetic DL-sphingosine were purchased from Miles-Yeda, Ltd., Kiryat Weizmann, Rehovoth, Israel. Phosphatidylserine, phosphatidylethanolamine, cardiolipin (diphosphatidylglycerol), and beef spinal cord sulfatide were obtained from Applied Science Laboratories, Inc. Phosphatidylinositol was bought from Pierce Chemical Co., Rockford, Ill. Glucosamine was obtained from Calbiochem, Los Angeles, Calif. All other carbohydrate standards were obtained from Pfanstiehl Labs., Inc., Waukegan, Ill. The fatty acid methyl ester standards for GLC were obtained from the Metabolism Study Section, National Heart Institute. Electronic grade hydrogen chloride gas was purchased from the Matheson Co., Inc., East Rutherford, N. J. Tri-Sil was bought from Pierce Chemical Co. Anisaldehyde was obtained from Eastman Organic Chemicals, Rochester, N. Y. The Evapo-Mix was obtained from Buchler Instruments, Inc., Fort Lee, N. J. Authentic gangliosides-G_{M1} and -G_{M2} were generously supplied by Dr. L. Svennerholm.

METHODS

Thin-Layer Chromatography

One-dimensional TLC was performed on plates of Silica Gel G. The nonpolar solvent system used for separation of neutral lipids (fraction I, Fig. 1) was petroleum ether–ethyl ether–acetic acid 90:10:1 (3). Glycerides were further separated into mono-, di-, and triglycerides employing ethylene dichloride–methanol 98:2 (4). The neutral glycolipids (fraction III, Fig. 1) were separated with chloroform–methanol–water 100:42:6 (5), and phospholipids from fractions III and IV (Fig. 1) with chloroform–methanol–water 65:25:4 (6). The gangliosides in the upper phase were separated by employing

[1]The frozen samples were generously supplied by the tissue procurement program, National Cancer Institute, Georgetown University, Contract No. PH 43-67-1134.

chloroform–methanol–water–ammonium hydroxide 60:35:6:2 (7). Two-dimensional TLC was performed on plates of Silica Gel F-254 using chloroform–methanol–ammonium hydroxide–water 180:105:7.5:7.5 in the first dimension and chloroform–methanol–ammonium hydroxide–water 120:160:5:5 in the second dimension (8). To visualize the lipids, the plates were sprayed with an anisaldehyde reagent (1 ml in 2 ml of sulfuric acid and 97 ml of glacial acetic acid), (9) and heated at 110°C for 10 min unless otherwise specified.

Sequential Lipid Analyses

The scheme followed for lipid analyses is shown in Fig. 1. The liver was blotted on filter paper to remove excess blood. Lipids were extracted from 0.5 to 1.0 g samples of liver by homogenizing the tissue with 20 ml of chloroform–methanol 2:1 in a Sorvall Omni-mixer. Residue was removed by filtration, and the filter cake was washed with an additional 30 ml of chloroform–methanol 2:1. The lipid extract was then partitioned and washed as described by Folch, Lees, and Sloane Stanley (10). Cholesterol, triglycerides, free fatty acids, glycolipids, and phospholipids were sequentially separated from the lower phase by silicic acid column chromatography (Fig. 1) employing the method of Vance and Sweeley (5), except where modifications are indicated.

From 30 to 90 mg of lipid were applied in chloroform to a 4 g silicic acid column. Cholesterol and glycerides were completely eluted with 150 ml of chloroform (fraction I, Fig. 1). Cholesteryl esters and cholesterol were separated by TLC using the nonpolar solvent system. The plates were stained with iodine vapor, the esters and free sterol were separately scraped from the plate and eluted with chloroform, and the cholesterol content was measured in a Technicon AutoAnalyzer by the ferric chloride method (11). Glycerides were measured by the fluorometric method of Kessler and Lederer (12). Free fatty acids were also demonstrable by TLC only in fraction I. The column was next eluted with 20 ml of ethyl acetate. This fraction II contained mainly yellow pigments and was discarded.

Acetone (400 ml) then completely removed all the mono-, di-, and trihexosyl ceramides from the column. Subsequent elution with 100 ml of acetone–methanol 9:1 removed tetrahexosyl ceramide (globoside). These eluates together formed fraction III (Fig. 1), which also contained cardiolipin and part of the phosphatidylinositol and ganglioside-G_{M3} (hematoside) (13). This pattern of elution was found to remove all glycolipids from the column and with less phospholipid contamination than was obtained by a single purge with 400 ml of acetone–methanol 9:1 (5). It also permitted the separation of tetrahexosyl ceramides from other neutral glycolipids.

Analysis of Neutral Glycolipids

Isolation. Following alkaline methanolysis (5) to remove cardiolipin and phosphatidylinositol, the neutral glycolipids, fatty acids, and fatty acid methyl esters were isolated as described by Vance and Sweeley (5), and were separated by preparative TLC. The compounds were detected by exposing the plates to either iodine vapor or bromthymol blue spray (10 mg/100 ml of 1.5 M NH_4OH). The separate glycolipids were then subjected to HCl methanolysis (5) for 24 hr. Fatty acid methyl esters from the glycolipids were extracted from the acidic methanolysate with hexane (5). These esters were identified on a Barber-Colman gas chromatograph equipped with a hydrogen flame ionization detector, using a 6 ft glass column, $^1/_8$ in. I.D., packed with EGS, 15%, at 175°C. The methanolysis mixture was then neutralized with silver carbonate by the method of Clamp, Dawson, and Hough (14). This procedure was found to remove HCl more conveniently and reliably than Amberlite chromatography (5). The galactosamine liberated during the methanolysis of the tetrahexosyl ceramides was *N*-acetylated with acetic anhydride (14) prior to quantitation by GLC.

Identification. All of the methyl glycosides were silylated with 100 μl of Tri-Sil. The resulting TMSi derivatives of methyl glycosides and added standard mannitol were separated on a Beckman GC-5 gas chromatograph equipped with an 8 ft steel column, $^1/_8$ in. I.D., and packed with SE-30, 3%, at 140°C. The carrier gas was high purity dry nitrogen. When the samples contained less than 0.01 μmoles of hexose, it was necessary to enhance the sensitivity of the chromatographic method by first removing all traces of pyridine from the silylation products under a stream of dry nitrogen. The TMSi-methyl glycosides were then dissolved in a small volume (25–50 μl) of hexane for GLC. The quantitative results were not affected by this procedure.

The area under GLC peaks was determined by triangulation. The response of the ionization detector to TMSi-mannitol was 1.21 times the detector response for equivalent amounts (moles) of TMSi-methyl glucoside. The amount of each glycolipid was calculated by assuming 1 μmole of glycolipid for each μmole of glucose.

Sphingosine. In several experiments, the methanolysis mixture was neutralized and taken to dryness under nitrogen. Methyl glycosides and methyl sphingosine were partitioned according to the method of Dawson and Sweeley (15). The sphingosine was determined by Sweeley's modification (16) of the method of Lauter and Trams (17).

Determination of Other Sugars. Hexosamines were released from the isolated purified glycolipids by acid hydrolysis and isolated according to the technique of Spiro (18). Glucosamine and galactosamine were

separated and quantified by the use of the amino acid analyzer by the method of Walborg and Ward (19). Sialic acid was determined by Warren's method (20). Total reducing carbohydrates were determined by the phenol–sulfuric acid method (21).

Phospholipids. Phosphorus was determined on the total lipid extract and on fractions I–IV by the technique of Bartlett (22). Although phosphorus amounting to 1.2–2.7% of the total in the lipid extract was found in fractions I and II, no phospholipids were demonstrated by TLC. From 3.7 to 13.1% (mean 10.0%) of the total lipid phosphorus was present in fraction III; the remainder was recovered from the silicic acid column in the final elution with 200 ml of methanol (fraction IV). The individual phospholipids were separated, and the relative amounts were determined in duplicate on a separate aliquot of the original total lipid extract by two-dimensional TLC (Fig. 4) (8).

Gangliosides. The methanol was removed from the upper phase of the original tissue extract under nitrogen, and the remaining water was removed by evaporation in a Buchler Evapo-Mix. The residue was taken up in 5–10 ml of water and dialyzed against 4 liters of water at 4°C for 18 hr. The dried dialysate was dissolved in 1 ml of water. Aliquots were taken for determination of sialic acid and separation of the individual gangliosides by one-dimensional TLC. From two separate liver extracts, larger amounts of material having the R_f of ganglioside-G_{M3} were prepared using preparative TLC in chloroform–methanol–water–ammonium hydroxide 60:35:6:2 (7). The appropriate sector was located with iodine vapor, scraped off, and eluted with chloroform–methanol–water 100:50:10. After HCl-methanolysis, the content of sphingosine, glucose, galactose, NANA, and fatty acid methyl esters were determined as outlined above.

RESULTS

Cholesterol, Glycerides, and Free Fatty Acids

The amounts of total cholesterol and glycerides in the 12 liver samples are shown in Table 1. Each value represents the average of at least four determinations. Total cholesterol varied from 2.0 to 5.4 mg/g wet weight with a mean of 3.9 ± 0.8. The percentage of cholesterol that was esterified (seven liver samples) varied from 19 to 32% (mean, 25%). The glyceride content varied widely, from 2.9 to 54.3 mg/g of wet tissue, with a mean of 19.4 ± 15.9 (Table 1). The glycerides in one sample (*B*) were separated by TLC and, as estimated qualitatively, the bulk was triglyceride.

Samples A, B, C, G, and H were extracted in duplicate, and fraction I (Fig. 1) was obtained from both extracts of each sample. The variance of the difference of the means for these paired duplicate extractions ranged from 0.003 to 0.013 mg/g wet weight for cholesterol and from 0.073 to 0.125 mg/g wet weight for glycerides. Free fatty acids were detectable in fraction I from each of nine livers examined by TLC (Fig. 2). No cholesterol, glyceride, or fatty acids were detectable by TLC in fraction II.

TABLE 1 NEUTRAL LIPIDS AND TOTAL PHOSPHOLIPIDS

Sample	Cholesterol	Glycerides	Phospholipids*
	mg/g wet weight		
A	3.9	4.8	21.0
B	3.9	17.7	23.8
C	3.7	2.9	28.7
D	4.4	24.9	23.5
E	5.4	54.3	25.9
F	4.1	4.0	26.4
G	4.1	39.6	24.3
H	3.3	20.7	22.4
I	3.6	9.5	24.4
J	3.9	20.8	23.1
K	4.2	28.5	26.4
L	2.0	5.1	30.7
Mean ± SD	3.9 ± 0.8	19.4 ± 15.9	25.1 ± 2.7

* Calculated by adding the phosphorus found in fractions III and IV and multiplying by 25.

Glycolipids

Isolation and Purification. The small amounts of phospholipids accompanying the neutral glycolipids in fraction III were completely removed by alkaline methanolysis (5) (Fig. 2). After the individual glycolipids were separated by preparative TLC and rechromatographed once, each was free of contamination by the others.

Composition and Analysis. The hexose in the monohexosyl ceramides isolated from each of the 12 livers was at least 95% glucose. The only other sugar detected was galactose. The dihexosyl ceramides contained Gal:Glc in a ratio of 0.90 to 1.01 in 10 of the 12 samples analyzed. In the ceramide trihexosides, the ratio of Gal:Glc obtained in a total of 10 livers varied from 1.41 to 2.03; in five, the ratio was between 1.86 and 2.03. Ceramide tetrahexosides examined in 10 samples had a ratio of Gal:Glc that ranged from 1.40 to 1.85.

Individual neutral glycolipids were isolated from 10 g of one liver. The content of sphingosine was measured, and the amount of hexose was determined by both GLC and the phenol–sulfuric acid method. The ratios of glucose to sphingosine obtained were the following: monohexosides, 1.10; dihexosides, 0.93; trihexosides, 1.10; and tetrahexosides, 1.10. The amounts of each glycolipid (μg/g wet weight) obtained by GLC and by the phenol–sulfuric acid methods, respectively, were the following: monohexosides, 10.1 and 10.6; dihexosides, 20.2 and 19.2; trihexosides, 11.9 and 7.5; and tetrahexosides, 11.5 and 9.2.

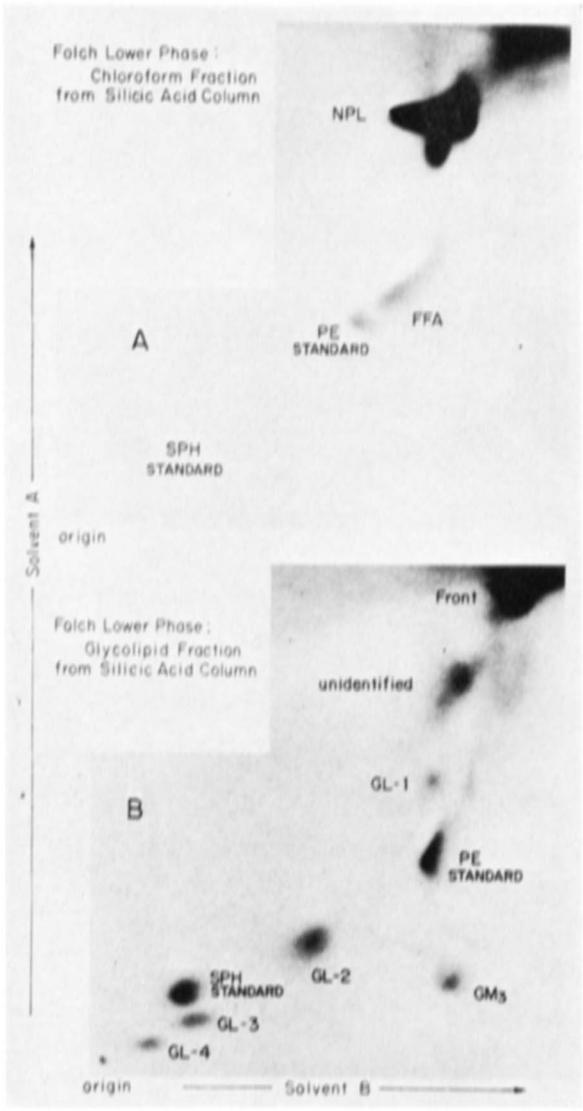

FIG. 2. TLC separations of fraction I (*A*) and fraction III (*B*). The equivalents of 5 mg of liver as fraction I and 250 mg of liver as fraction III were applied. The solvent systems used were chloroform–methanol–ammonium hydroxide–water 180:105:7.5:7.5 (solvent A), and chloroform–methanol–ammonium hydroxide–water 120:160:5:5 (solvent B).

Since recovery of the *N*-acetylated galactosamine by GLC was poor, determination of the glucosamine and galactosamine in trihexosides and tetrahexosides was performed on the amino acid analyzer. The trihexosides contained no detectable hexosamines. Tetrahexosides contained only galactosamine, in a 1:1 ratio with glucose. Only trace amounts of sialic acid were found in any of the neutral glycolipids. In each of the neutral glycolipids, $C_{18:0}$ and $C_{16:0}$ together constituted 60–75% of the normal fatty acids. The $C_{22:0}$, $C_{24:0}$, and $C_{24:1}$ acids each represented less than 10% of the total. The analysis did not include any assessment of possible α-hydroxy fatty acids.

Ganglioside-G_{M3}. The fraction III obtained from extracts of all livers contained a phosphorus-free compound which migrated on TLC to a position between the origin and the tetrahexosyl ceramides (Fig. 3) and below phosphatidylethanolamine on two-dimensional TLC (Fig. 2). A sample of this material obtained from the extract of 10 g of one liver contained sphingosine, Glc, Gal, and NANA in a ratio of 1:1:1:0.7. The relative amounts of NANA were probably reduced by loss on silicic acid chromatography (23). There was no detectable glucosamine or galactosamine. The sialic acid was shown to be NANA rather than *N*-glycolyl neuraminic acid by paper chromatography (24). The predominant fatty acid methyl esters present in the hexane extract of the acidic methanolysate were $C_{16:0}$ and $C_{18:0}$. The compound was, therefore, tentatively identified as ganglioside-G_{M3} or hematoside. The quantity of hematoside in the lower phase of 10 livers varied from 0.01 to 0.04 μmoles/g wet weight with a mean of 0.02 μmoles (SD $\pm$ 0.008).

Quantification of Neutral Glycolipids. The reproducibility of the gas chromatographic analyses of TMSi-methyl glycosides in normal human liver is presented in Table 2. This represents all the glycolipids in fraction III. Synthetic *N*-lignoceryl DL-sphingosine βD-lactoside (0.74 μmoles) was added to a liver extract previously determined to contain 0.21 μmoles of endogenous dihexosides. The recovery of total dihexosides was 92% of theoretical.

The amounts of each neutral glycolipid present in all 12 livers is presented in Table 3. Ceramide dihexosides were usually present in the greatest amounts, but in three livers (C, F, and K), the amount of monohexosides slightly exceeded that of the dihexosides. Ceramide

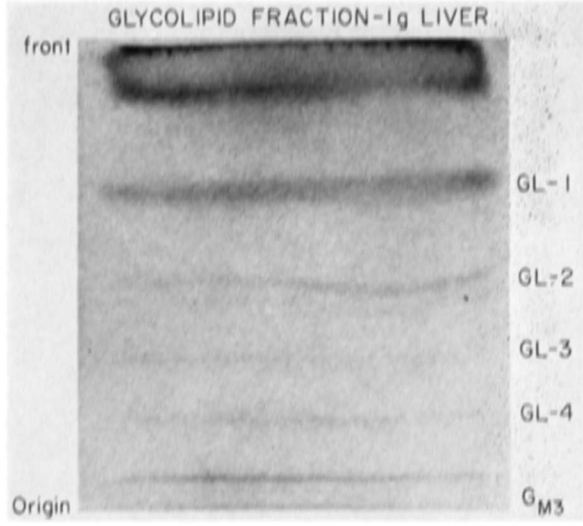

FIG. 3. Preparative TLC of the glycolipids in fraction III representing 1 g of liver. The solvent used was chloroform–methanol–water 100:42:6 (5). The glycolipids were visualized with iodine vapor.

TABLE 2 REPRODUCIBILITY OF GLC DETERMINATIONS OF GLYCOLIPIDS

	GL-1	GL-2	GL-3	GL-4	G_{M3}	Total
			μmoles/g wet weight			
Sample G						
Extraction 1	0.03	0.04	0.02	0.01	0.02	0.12
Extraction 2	0.02	0.04	0.02	0.01	0.02	0.11
Sample H						
Extraction 1	0.02	0.03	0.02	0.01	0.02	0.10
Extraction 2	0.03	0.03	0.02	0.01	0.01	0.10

trihexosides and tetrahexosides were present in lesser amounts. The mean total glycolipid content in the 12 livers was 0.19 μmoles/g wet weight with SD ± 0.11 μmoles/g wet weight. Data suggesting that the amounts of the individual and total neutral glycolipids in liver from females are higher than those in males is also shown in Table 3.

Sulfatide

A sulfatide standard (beef spinal cord sulfatide) was also chromatographed on silicic acid. It was eluted from the column in fraction III. Sulfatide migrated with the trihexosides on TLC when the standard glycolipid solvent was employed. The fraction III obtained from liver L was also chromatographed on DEAE cellulose according to the method of Svennerholm and Thorin (25). We could detect no sulfatide in the lithium chloride fraction, although the entire fraction was concentrated and chromatographed on TLC. The amount of sulfatide in normal liver is apparently very small (26).

Phospholipids

The mean content of total phospholipid in the 12 livers was 25.1 ± 2.7 (SD) mg/g wet weight, with a range of 21.0–30.7 (Table 1). This range was greater than the mean variation of phospholipid content found in duplicate extracts of five different livers (mean 4.5%). The distribution of the phospholipids as obtained by two-dimensional TLC (Fig. 4) is shown in Table 4.

Cardiolipin and phosphatidylinositol comprised most of the phospholipid found in fraction III. Authentic

TABLE 3 NEUTRAL GLYCOLIPIDS IN LIVERS FROM MALES AND FEMALES

	Sample	Age	GL-1	GL-2	GL-3	GL-4	Total
		yr			*μmoles/g wet weight*		
Female	A	4	0.07	0.21	0.09	0.06	0.43
	B	8	0.07	0.13	0.10	0.06	0.36
	C	65	0.08	0.07	0.04	0.04	0.23
	D	58	0.05	0.06	0.02	0.03	0.16
	E	41	0.03	0.08	0.03	0.01	0.15
	F	55	0.07	0.06	0.04	0.04	0.21
Mean			0.06 ± 0.02	0.10 ± 0.06	0.05 ± 0.03	0.04 ± 0.02	0.26 ± 0.11
Male	G	25	0.03	0.04	0.02	0.01	0.10
	H	21	0.03	0.03	0.02	0.01	0.09
	I	55	0.06	0.06	0.02	0.02	0.16
	J	22	0.01	0.06	0.02	0.02	0.11
	K	44	0.04	0.03	0.02	0.02	0.11
	L	8	0.04	0.07	0.03	0.02	0.16
Mean			0.04 ± 0.02	0.05 ± 0.02	0.02 ± 0.004	0.02 ± 0.01	0.12 ± 0.03
Mean (female and male)			0.05 ± 0.02	0.08 ± 0.05	0.04 ± 0.03	0.03 ± 0.02	0.19 ± 0.11
Difference							
"*t*" (Student's "*t*" test)			2.6520	2.1211	2.3143	2.9066	2.8146
P			$0.05 > P > 0.02$	$0.1 > P > 0.05$	$0.05 > P > 0.02$	$0.02 > P > 0.01$	$0.02 > P > 0.01$

TABLE 4 PERCENTAGE* DISTRIBUTION OF PHOSPHOLIPIDS IN NORMAL HUMAN LIVER

Phospholipid	Sample B	C	F	G	H	M	N	Mean ± SD
PC	35.4	41.2	49.5	41.3	44.9	44.0	42.0	42.6 ± 4.3
Lyso PC	1.4	1.4	0.0	2.2	1.9	1.0	1.4	1.3 ± 0.7
PE	25.4	29.5	24.7	25.1	25.8	24.4	22.9	25.4 ± 2.0
PI	9.7	3.4	8.2	4.9	3.9	8.8	9.6	6.9 ± 2.7
PS	4.6	11.0	3.6	10.8	9.9	6.1	6.5	7.5 ± 3.0
SPH	7.3	5.4	6.4	6.5	6.4	9.9	7.0	7.0 ± 1.4
CL	3.4	2.8	3.3	5.9	5.1	2.7	4.0	3.9 ± 1.2
Origin	0.0	1.2	1.9	0.8	0.3	0.8	1.2	0.9 ± 0.6
Front unidentified	12.8	4.2	2.7	2.7	1.8	2.4	5.9	4.6 ± 3.9

* μgP/total μgP × 100.

cardiolipin and the material from liver cochromatographing with it (Fig. 4) were completely eluted from the silicic acid column in the combined acetone and acetone–methanol 9:1 eluates representing fraction III and in the lithium chloride fraction from a DEAE column (27). This liver phospholipid also proved to be completely labile to alkaline hydrolysis and alkaline methanolysis. Fatty acids and water-soluble phosphorus were identified among the products. This cardiolipin constituted an average of 3.9% of the total liver phospholipid.

Both authentic phosphatidylinositol and the liver phospholipid migrating with it on TLC appeared in the acetone eluate, and continued into the acetone–methanol and methanol (fraction IV) eluates. A small amount of unidentified phospholipid in fraction III migrated between the monohexosides and dihexosides on TLC.

Gangliosides

The upper phase of the original extract of six different liver samples contained an average of 41.4 μg of sialic acid per g wet weight, the range being 18.0–59.3. As judged from TLC, G_{M3}(hematoside) was the predominant ganglioside, and there were small amounts of gangliosides-G_{M2} and -G_{M1}. Also present was material having a slightly greater R_f than G_{M3} and staining blue-green with anisaldehyde. Larger quantities of this unknown compound and ganglioside-G_{M3} were obtained by preparative TLC. The ganglioside-G_{M3} contained sphingosine, glucose, galactose, and NANA in equimolar amounts. The unknown compound contained no carbohydrate detectable by GLC, sphingosine, NANA, or phosphorus. The content of G_{M3} in the upper phase of two samples (D and J) was 0.03 μmoles and 0.05 μmoles/g wet weight, respectively.

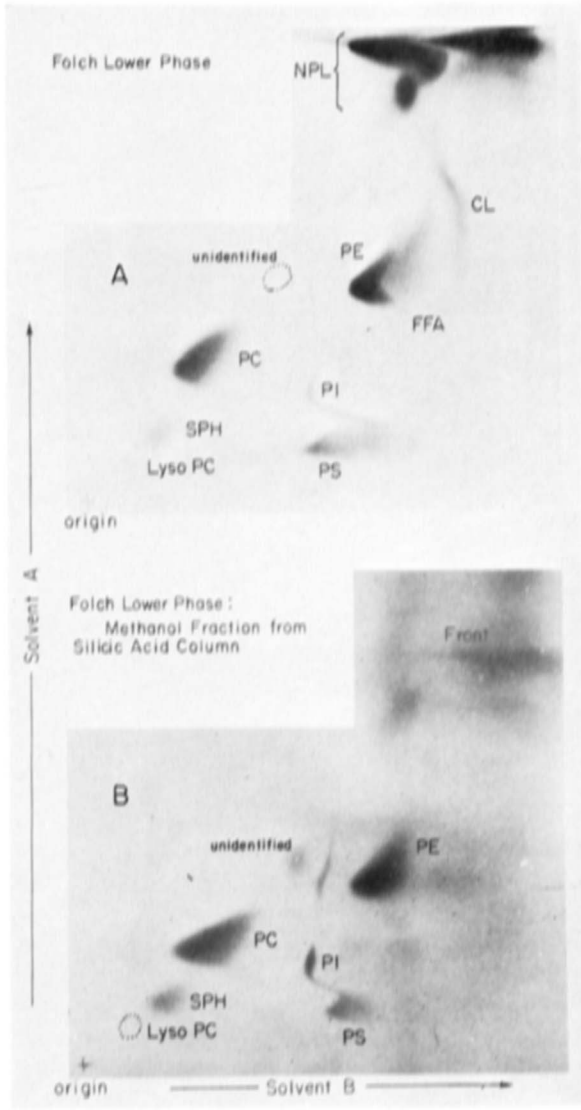

FIG. 4. TLC separation of the lower phase of the Folch extract (*A*) and of fraction IV (*B*). The equivalents of 10 mg of liver as lower phase and of 12.5 mg of liver as fraction IV were applied. The solvent systems used were chloroform–methanol–ammonium hydroxide–water 180:105:7.5:7.5. (solvent A), and chloroform–methanol–ammonium hydroxide–water 120:160:5:5 (solvent B).

DISCUSSION

The present data indicate that the total quantity of neutral glycolipid in the human liver varies from 0.09 to 0.43 μmoles/g wet weight (0.1–0.4 mg/g, assuming an average mol wt of 1000). These values are higher than the single other figure in the literature of 0.4 mg/g dry weight (26). The concentration of glycolipid in the human liver is thus considerably lower than that in spleen (28) or kidney (29).

Information concerning the distribution and composition of the neutral glycolipids in various tissues has recently been summarized (30). Our observation that the monohexosyl ceramides in normal human liver are primarily glucosyl ceramides agrees with the findings of Svennerholm and Svennerholm (2). In this regard the liver is comparable to the spleen (2) and plasma (2, 5). For most of the samples analyzed, we found, in agreement with a previous report (2), that dihexosyl ceramides were the most abundant of the neutral glycolipids in liver. In several of our samples, however, the content of monohexosides was greater.

The relatively small amounts of trihexosides and tetrahexosides in normal liver permitted only tentative identification of their precise structures. One possible explanation for the low ratio of Gal:Glc obtained in the trihexosides might be the presence of a mixture of compounds having the structures Gal-Gal-Glc-Cer and Gal-Glc-Glc-Cer. This is unlikely, however, because the ratio of sphingosine to glucose in the trihexosides was very close to unity. The stoichiometry of sphingosine,

glucose, galactose, and galactosamine found in the tetrahexosides is consistent with the structural sequence GalNAc-Gal-Gal-Glc-Cer, as has been found in globosides of erythrocytes (31).

Ganglioside-G_{M3} or hematoside, first described in horse erythrocytes (32, 33), is the major ganglioside in human spleen (34). Suzuki has reported the presence of hematoside in a single human liver (35). Suzuki, Suzuki, and Kamoshita have found that hematoside represents not less than 80% of the gangliosides in liver (36). Several of the other gangliosides contain more than one sialic acid residue per molecule (36). This at least partially explains why the amount of hematoside in the upper phase of the liver extract is lower than that which would be predicted on the basis of the amount of sialic acid present. We have also found G_{M3} to be the predominant ganglioside in all of the normal livers examined. Our analyses are compatible with the structure of liver hematoside as being Gal(NANA)-Glc-Cer, but the sequence of the carbohydrate moieties was not determined.

Our data are in reasonable agreement with other reports of the cholesterol (37), cholesteryl ester (38, 39), glyceride (40, 41), and phospholipid (42) content of human liver. Cardiolipin has previously been reported to be present in one sample of human liver (43). A sample of liver E (Table 1) was reexamined histologically because its glyceride content was unusually high. Some vacuolization, viewed in H and E preparations, was interpreted as representing moderate fatty infiltration. If the values from liver E are omitted from the compilation in Table 1, the mean glyceride and cholesterol concentrations are 16.2 ± 12.0 and 3.7 ± 0.2, respectively.

The neutral glycolipids represent an extremely small fraction (about 0.4%) of the total lipids normally present in liver. They thus require special care in each step of the procedures designed to quantify them. The phenol–sulfuric acid method is a much simpler method than is GLC for determining the quantity of neutral sugars in glycolipids. We found, however, that the phenol–sulfuric acid method gave values for the sugars hydrolyzed from the glycolipids that were 10–30% lower than those obtained by GLC.

An estimation of the possible contribution of entrapped blood to the glycolipid content of the liver may be made. Although as much as 13% wet weight of liver has been attributed to blood (44), a more reasonable estimate would seem to be not more than 5%. Employing this assumption, and the data of Vance and Sweeley (5) on the glycolipid content of plasma and packed blood cells, we estimate the contribution of blood glycolipids to the total present in liver to be 1, 1, 3, and 10% of the mono-, di-, tri-, and tetrahexosyl ceramides, respectively.

In Table 5 is provided a summary of the amounts of liver required for determination of an abnormality in the concentration of most of the lipids involved in known lipid-storage diseases. Although much smaller amounts of tissues permit qualitative identification of a gross increase in the content of a specific lipid, 0.5–1 g of liver should be obtained at biopsy to permit a complete examination for lesser changes in lipid content.

TABLE 5 AMOUNTS OF LIVER REQUIRED FOR QUANTITATIVE LIPID ANALYSES

Lipid	Mean Concentration in Human Liver	Amount of Lipid Required for Analysis	Amount of Tissue (wet weight)
	μg/mg	*μg*	*mg*
Total cholesterol	4	60	15
Esterified cholesterol	1.0	60	60
Total glyceride	20	60	3
Total phospholipids	25	25	1
Phospholipid distribution	25	250	10
Total sialic acid	0.04	2	50
Total neutral glycolipids*	0.20	80	400

* The amounts of liver shown are those that are desired for a single accurate determination of the concentration of a given lipid using the methods employed in the text.

The skilled technical assistance of Miss Seniye Temel and Mrs. Barbara Davis is gratefully acknowledged.

Manuscript received 29 December 1969; accepted 17 March 1970.

REFERENCES

1. Fredrickson, D. S. 1968. *Pathol. Eur.* **3:** 121.
2. Svennerholm, E., and L. Svennerholm. 1963. *Nature (London).* **198:** 688.
3. Mangold, H. K., and D. C. Malins. 1960. *J. Amer. Oil Chem. Soc.* **37:** 383.
4. Jatzkewitz, H., and E. Mehl. 1960. *Hoppe-Seyler's Z. Physiol. Chem.* **320:** 251.
5. Vance, D. E., and C. C. Sweeley. 1967. *J. Lipid Res.* **8:** 621.
6. Wagner, H. 1960. *Fette Seifen Anstrichm.* **62:** 1115.
7. Wherrett, J. R., and J. N. Cumings. 1963. *Biochem. J.* **86:** 378.
8. Fredrickson, D. S., H. R. Sloan, and C. T. Hansen. 1969. *J. Lipid Res.* **10:** 288.
9. Waldi, D. 1965. *In* Thin-Layer Chromatography. E. Stahl, editor, Springer-Verlag, New York Inc., New York. 485.
10. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. *J. Biol. Chem.* **226:** 497.
11. Technicon Instruments, Total Cholesterol Procedure N24a. 1964. *In* Auto Analyzer Manual. Technicon Corporation, Chauncey, N. Y.
12. Kessler, G., and H. Lederer. 1966. *In* Automation in Analytical Chemistry. L. T. Skeggs, Jr., editor. Mediad Inc., New York. 341.
13. Svennerholm, L. 1964. *J. Lipid Res.* **5:** 145.
14. Clamp, J. R., G. Dawson, and L. Hough. 1966. *Biochem. J.* **100:** 35c.

15. Dawson, G., and C. C. Sweeley. 1970. *J. Biol. Chem.* **245:** 410.
16. Sweeley, C. C. 1963. *J. Lipid Res.* **4:** 402.
17. Lauter, C. J., and E. G. Trams. 1962. *J. Lipid Res.* **3:** 136.
18. Spiro, R. G. 1966. *In* Methods of Enzymology. E. F. Neufeld and V. Ginsburg, editors. Academic Press Inc., New York. **8:** 19.
19. Walborg, E. F., Jr., and D. N. Ward. 1963. *Biochim. Biophys. Acta.* **78:** 304.
20. Warren, L. 1959. *J. Biol. Chem.* **234:** 1971.
21. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. *Anal. Chem.* **28:** 350.
22. Bartlett, G. R. 1959. *J. Biol. Chem.* **234:** 466.
23. Wolfe, L. S., and J. A. Lowden. 1964. *Can. J. Biochem.* **42:** 1041.
24. Spiro, R. G. 1960. *J. Biol. Chem.* **235:** 2860.
25. Svennerholm, L., and H. Thorin. 1962. *J. Lipid Res.* **3:** 483.
26. Svennerholm, L. 1966. *Cited in* Martensson, E. Glycolipids of Human Kidney. Ph.D. Thesis. Goteborg, 16.
27. Rouser, B., G. Kritchevsky, and A. Yamamoto. 1967. *In* Lipid Chromatographic Analysis. G. V. Marinetti, editor. Marcel Dekker, Inc., New York. 120.
28. Suomi, W. D., and B. W. Agranoff. 1965. *J. Lipid Res.* **6:** 211.
29. Martensson, E. 1966. *Biochim. Biophys. Acta.* **116:** 296.
30. Martensson, E. 1969. *In* Progress in the Chemistry of Fats and Other Lipids. R. T. Holman, editor. Pergamon Press, Inc., New York. **10,** Pt. IV: 367–407.
31. Yamakawa, T., S. Yokoyama, and N. Handa. 1963. *J. Biochem.* (*Tokyo*). **53:** 28.
32. Yamakawa, T., and S. Suzuki. 1951. *J. Biochem.* (*Tokyo*). **38:** 199.
33. Klenk, E., and G. Padberg. 1962. *Hoppe-Seyler's Z. Physiol. Chem.* **327:** 249.
34. Svennerholm, L. 1963. *Acta Chem. Scand.* **17:** 860.
35. Suzuki, K. 1968. *Science.* **159:** 1471.
36. Suzuki, K., K. Suzuki, and S. Kamoshita. 1969. *J. Neuropathol. Exp. Neurol.* **28:** 25.
37. Kritchevsky, D. 1958: *In* Cholesterol. John Wiley & Sons, Inc., New York. 281.
38. Man, E. B., B. L. Kartin, S. H. Durlacher, and J. P. Peters. 1945. *J. Clin. Invest.* **24:** 623.
39. Ralli, E. P., S. H. Rubin, and S. Rinzler. 1941. *J. Clin. Invest.* **20:** 93.
40. Lieber, C. S., and N. Spritz. 1966. *J. Clin. Invest.* **45:** 1400.
41. Farquhar, J. W., R. C. Gross, R. M. Wagner, and G. M. Reaven. 1965. *J. Lipid Res.* **6:** 119.
42. Fredrickson, D. S. 1966. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill Book Co., New York. 603.
43. Rouser, G., G. Kritchevsky, A. Yamamoto, A. G. Knudson, Jr., and G. Simon. 1968. *Lipids.* **3:** 287.
44. Brückmann, G., and S. G. Zondek. 1939, *Biochem. J.* **33:** 1845.